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PRINCIPAL INVESTIGATOR: Jasmine V. Abella

CONTRACTING ORGANIZATION: McGill University
                                                    Montreal Quebec H3A 1A1 Canada

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14. ABSTRACT The Hepatocyte Growth Factor (HGF) RTK, Met, regulates cell proliferation, differentiation, migration, invasion and survival. Met activation is tightly controlled through several levels of regulation to achieve an appropriate biological response. In addition to mutations that activate the Met receptor in human cancer, I have previously shown that the specific uncoupling of Met from ubiquitination results in its oncogenic activation through deregulate endocytosis. My recent work has uncovered a novel role for the Gab1 scaffold in regulating Met internalization and subsequent degradation. HGF stimulation induces membrane ruffling events including the formation of lamellipodia and circular dorsal ruffles (CDRs). I show that Gab1 localizes to CDRs and recruits the Met receptor to this plasma membrane compartment where receptors are then internalized into the cell. Overexpression of Gab1 in HeLa and MDCK cells increases CDR formation, induces a more rapid Met translocation to a perinuclear compartment and enhances HGF induced Met degradation. Mutations in Gab1 that impair recruitment to Met or the plasma membrane, fail to enhance Met degradation. Interestingly, we also show that the endocytic protein, STAM2, can associate with Gab1 and is enriched in CDRs with Gab1. This represents a novel function for both proteins in normal Met downregulation.

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Oncogenic receptor activation, modes of receptor internalization, Deregulated receptor degradation, Circular Dorsal Ruffle formation.

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INTRODUCTION

The Met receptor tyrosine kinase (RTK) and its ligand, Hepatocyte growth factor (HGF) are deregulated in human breast cancer, through over-expression, the acquisition of point mutations that elevate the Met receptor catalytic activity and co-expression of the ligand and receptor at the invasive tumour margins. We have shown that HGF stimulates the dissociation, motility and invasion of human breast cancer cell lines in culture. These are all processes that correlate with enhanced tumor growth and invasion. Deregulation of the Met receptor was generally thought to result in ligand-independent activation of the receptor, however, we have recently shown that escape from down-regulation constitutes another mechanism leading to RTK deregulation and that this mechanism may be common in human breast cancer (1).

Under normal conditions, Met downregulation involves receptor activation, ubiquitination, clathrin dependent endocytosis and degradation (2-4). I have demonstrated that receptor ubiquitination is not required for internalization, but is a signal necessary for efficient degradation (2). My next aim was to determine the requirements for Met receptor internalization and examine whether these events are deregulated in breast cancer. Studies on the EGFR (epidermal growth factor receptor) have clearly demonstrated that several internalization pathways are utilized by this receptor (5), However, it is not clear how each pathway regulates receptor signaling and degradation. These studies have not yet been carried out for the Met receptor, therefore it will be important to establish whether Met can internalize through clathrin-independent pathways, how they impact on Met signaling and stability and finally whether certain pathway(s) are selected for in Met overexpressing breast cancers.

BODY

(1) Identification of a novel mechanism of Met receptor downregulation.

I have previously shown that ubiquitination of the Met receptor is required for proper receptor degradation but is not essential for internalization. I hence sought to identify whether any of established Met binding proteins could play a role in regulating Met internalization. Using a structure function approach, I established T47D breast cancer epithelial cell lines expressing Met receptor mutants uncoupled from Grb2 and Cbl, two proteins known to be essential for in internalization of the EGFR (Epidermal Growth Factor Receptor). However, confocal microscopy studies of these mutant Met receptors demonstrated no defect in internalization upon HGF stimulation (Abella *et. al.* unpublished data Fig 1A). Similar results were obtained with siRNA depletion of either proteins (Abella *et. al.* unpublished data).

Upon HGF stimulation another scaffolding protein, Gab1 (Grb2 associated binder 1) is rapidly phosphorylated, recruited to the Met receptor and is responsible for propagating Met signalling to the PI3K, MAPK and Crk dependent pathways (6). Gab1 recruitment to Met is both indirect, by binding through Grb2 and direct, by binding to Y1349 in the C-terminus of Met. The direct recruitment of Gab1 to an RTK is specific to Met as it is only recruited indirectly to the EGFR through Grb2 (7). Interestingly, overexpression of Gab1 in both HeLa and MDCK (canine kidney epithelial) cells enhances HGF induced Met degradation (Fig 2A and C). Mutations in Gab1 impairing its recruitment to Met or the plasma membrane, fail to enhance Met degradation (Fig 2B). This data proposes a novel role for Gab1 which has only been studied downstream from Met in the context of a positive regulator. Gab1 enhanced Met degradation requires HGF activation of the Met receptor indicating that both normal

phosphorylation of the receptor and Gab1 recruitment is required. As a control to ensure that Gab1 does indeed enhance Met degradation and does not simply induce Met translocation to a detergent insoluble compartment, cells with or without Gab1 over expression were harvested under boiling lysis conditions, solubilizing all cellular compartments. Under these conditions, enhanced Met degradation was still observed (Fig 2D). Furthermore, Gab1 enhanced met degradation is dependent on HGF stimulation and not on the level of Gab1 overexpression (Fig 2E). Since I have previously shown that Met ubiquitination is required for efficient degradation of the receptor, I addressed whether the enhanced Met degradation induced by Gab1 was due to elevated Met ubiquitination. Conversely however, Gab1 over expression reduced levels of Met receptor ubiquitination (Fig 2F).

To determine whether Gab1 over expression altered Met trafficking in HeLa cells, confocal microscopy studies of Met upon HGF stimulation was performed. This revealed that over expression of Gab1 induced a more rapid translocation of Met to a perinuclear compartment (that is also poitive for the early endosome marker, EEA1) by 30 minutes, whereas localization of the receptor in vector control cells required at least 1 hour of HGF stimulation to reach this compartment (Fig 3). Due to this alteration is trafficking, I will establish if Gab1 enhanced Met degradation is a lysosomal and/or proteosomal dependent process using established inhibitors of the lysosome (concanamycin A) and the proteosome (lactacystin).

A previous report indicated that Gab1 localizes to early endosomes (Rab4 and 5 positive) by subcellular fraction, downstream from an EGFR mutant (8). However, in our lab, HA or GFP tagged Gab1 has never been visualized on any endosomal structures, but rather, localization is limited to the plasma membrane, particularly to lamellipodia (9). This would suggest that Gab1 does not internalize with the Met receptor on endosomes, but functions at the level of the plasma membrane to either enhance Met internalization through the clathrin dependent pathway or alternatively may induce internalization through another mechanism. Confocal microscopy of Gab1 and clathrin revealed that these proteins do not co-localize at the plasma membrane, suggesting that Gab1 may be inducing Met internalization through a clathrin independent manner (data not shown). Work by McNiven's group has recently shown that 'large scale' internalization of the EGF (epidermal growth factor) and PDGF (platelet derived growth factor) receptors can occur through a process known as circular/dorsal ruffles , CDRs (10). CDR formation is a process that initiates a circular membrane protrusion from the apical surface within minutes of growth factor stimulation and collapse back onto the cell surface, much like wave. These structures can occur near the leading edge of a cell and may function to induce remodeling of the static actin cytoskeleton to enable lamellipodia formation and cell migration. However, waves and lamellipodia can occur independently of one another. The eventual collapse of a CDR can induce internalization of up to 50% of the total number of EGFR present on the cell surface within minutes of EGF stimulation, as shown by live cell imaging (11). Although CDRs have been viewed as a form of macropinocyctosis and share some of the same components (actin, Rac,Pak1, WAVE2, PI3K), there are several differences that distinguish these processes. For example, growth factor stimulation induces macropinosomes to pinch off rapidly and continuously from the plasma membrane and do not appear to recruit RTKs. Dorsal ruffles on the other hand, form once, persisting for up to 20 minutes and are rich in RTKs and proteins such as dynamin, cortactin, Rab5 and RN-tre, which have not been shown to be involved in macropinosome formation (12). How RTKs are selectively sequestered on CDRs remains unknown.

Given the time frame in which CDRs occur and that they are capable of inducing bulk receptor internalization, Met internalization through these structures could explain the more rapid localization of Met to a perinuclear compartment observed in Gab1 over expressing cells. As HGF has been shown to induce dorsal ruffles, particularly in MDCK cells (13), I am currently investigating the hypothesis that

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Met can internalize through dorsal ruffles and that Gab1 is required to mediate this process for Met. To examine whether Gab1 is present in CDRs, MDCK cells stably expressing GFP Gab1 were stimulated with HGF and imaged using both live cell and fixed cell confocal microscopy. Within less than 5 minutes of HGF stimulation, MDCK cells form CDRs which are positive for GFP Gab1 (Fig 4A). In HeLa cells which do not normally form dorsal ruffles, over expression of GFP Gab1 induces CDR formation (Fig 4B). Quantification of the number of cells which form dorsal ruffles by staining for actin in wt and GFP Gab1 stable MDCK cells showed that Gab1 increased the number of cells that form f dorsal ruffles by 4.3 fold and increase the time frame in which they occur (Fig 4C and data not shown). Notably, a Gab1 mutant deficient in membrane targeting (Gab1ΔPH) and is unable to enhance Met degradation, does not localize to dorsal ruffles (Frigault *et. al.* unpublished data). I have now established HeLa and MDCK cell lines with stable knock down of Gab1 with an shRNA targeting Gab1, subcloned into a GFP containing retroviral plasmid (LMP vector). The MDCK cell line will be examined for its ability to form dorsal ruffles upon HGF stimulation, and if no defect is observed, I will address whether Met is still able to localize to these structures deficient in Gab1. The rate of Met receptor degradation will also be assayed in both MDCK and HeLa Gab1 stable knock down lines.

Preliminary studies of Met and Gab1 on fixed cell samples, show that Met is present on Gab1 positive CDRs but Met distribution appears to be restricted to specific 'domains' on the ruffle (Fig 4B and C, see arrows). Live cell imaging will allow me to visualize Met internalization in real time and determine whether Met is restricted to specific domains on the ruffle or whether this is an artifact of fixation. In addition to using our wide field live cell imaging microscope, I have recently set up the conditions for live cell imaging on our confocal microscope which has provided us with much higher resolution. MDCK cells stably expressing Met with an GFP N-terminal fluorescent tag with and without co-expression of mCherry Gab1 are currently being established to perform live cell imaging on.

The serine kinase Pak1 (p21 activated kinase) has been shown to be required for the formation of CDRs and has been observed on vesicles which pinch off from the dorsal ruffle after it has collapsed back onto the plasma membrane (14). These Pak1 vesicles are EEA1 positive and connect into the endocytic pathway (15). Recently, another student in the lab performed a mass spectrometry proteomics study to identify new Met binding proteins using a Tap-tag Met construct (Paliouras *et. al.* unpublished data). Pak1 was identified as a Met associated protein and I have since validated this interaction in 293 cells over expressing Met and mycPak1 (Fig 5A). As I have demonstrated that Gab1 is present on dorsal ruffles, I next addressed if Gab1 can also form a complex with Pak1 and whether this is Met dependent. As shown in figure 5B, Gab1 is able to associate with Pak1 and this interaction does not appear to require Met activation (check). Similarly to Gab1 over expression, preliminary data suggests that Pak1 over expression, which induces circular ruffle formation, is also able to enhance Met receptor degradation (Fig 5C).

Given that I do not observe Gab1 on any endosomal structures and in light of our new finding that both Met and Gab1 can associate with Pak1, I am currently examining whether Met co-localizes with Pak1 positive vesicles, which have been shown to be derived from a Pak1 positive dorsal ruffle (14). This will be performed using both exogenous expression of GFP Pak1 and using an antibody to endogenous Pak1. Similarly, the extent of Met co-localization with Pak1 will be determined in HeLa and MDCK cells with or without Gab1 over expression to address whether Gab1 enhances co-localization. MDCK stable cell lines expressing GFP Pak1 and mCherry Met are currently being established for live cell imaging. This method will allow me to directly follow the formation of a circular ruffle, its collapse and any production of vesicles that may pinch off into the cell thereafter.

RTK internalization through circular dorsal ruffles would represent and efficient and fast mechanism to rapidly degrade receptors. This would likely impact on cell growth and transformation, hence, one

would predict that such a pathway could be selected against in cancer development. Indeed, CDR formation in pancreatic and prostate tumor cell lines was reported to be severely decreased compared to primary human fibroblasts (refs). Similarly, actinin-4, an actin binding protein involved in CDR formation, was found to be expressed at significantly lower levels in prostate cancer cell lines compared to non-cancerous basal cells (refs). Restoration of actinin-4 levels in the cancer derived cell lines inhibited cell proliferation. It would therefore be important to establish whether Met positive breast cancer cell lines are deficient in CDR formation.

These data are currently being written up for a manuscript and will be submitted by the end of April 2007.

(2) Determine whether the naturally occurring Met juxtamembrane (JM) mutation T992I, identified in human breast cancer exhibits altered signalling and trafficking.

I have previously established T47D cell populations stably expressing a Met T992I mutant receptor to study Met ubiquitination and trafficking. However, when assaying ubiquitination of mutant receptor, I saw no difference in the level ubiquitination as compared to the wt receptor. Similarly, confocal microscopy studies revealed no defect in receptor internalization or trafficking caused by the juxtamembrane mutation. Finally, a postdoctoral fellow in our lab has also demonstrated that the Met T9921 mutant receptor does not exhibit altered kinase activity compare to the wt receptor. Therefore, I have decided not to go further with this project.

(3) A novel function for the endocytic adaptor proteins Hrs and STAM on circular ruffles.

The Hepatocyte Growth Factor Receptor Tyrosine Kinase Substrate, Hrs (also called Hgs) and its binding partner STAM, Signal Transducing Adaptor Molecule, function at the level of the sorting endosome to recognize ubiquitinated cargo, such as RTKs and facilitate their internalization into the endosomal lumen, committing them for lysosomal degradation (16). Hrs contains a FYVE domain which binds to phosphatidylinositol-3-phosphate (PI3P), targeting Hrs and consequently STAM, to the endosomal membrane. Association between Hrs and STAM requires the coiled coil domains of both proteins. STAM contains an atypical SH3 domain which recognizes PX(V/I)(D/N)RXXKP motifs, and through this SH3 domain, can recruit one of two deubiquitinating enzymes, UBPY or AMSH, both of which contain this atypical proline rich motif (Fig 6 model) (17). Our lab has previously demonstrated that Gab1 contains an atypical proline rich motif which is recognized and bound by an SH3 domain of Grb2 (18). I therefore decided to test whether the atypical proline rich motif (PPVDRNLKP) of Gab1 was also a substrate for STAM's SH3 domain. As shown in figure 6B and C, Gab1 is able to associate with STAM and this interaction is dependent upon STAM's SH3 domain and the atypical proline rich motif 5 in Gab1. The interaction between STAM and Gab1 did not require Met induced phosphorylation of Gab1, in agreement with a proline-SH3 mode of binding (Fig 6B). An interaction between endogenous STAM and Gab1 could also be seen in HeLa cells (Fig 6D). This association appears to be weak prior to HGF stimulation, becomes enhanced with stimulation and disappears after 30 minutes. To determine where Gab1 and STAM associate in the cell since Gab1 localizes to the plasma membrane and STAM has only been reported to localize to endosomes, I performed confocal microscopy on HeLa cells transfected with GFP Gab1 and HA STAM. Interestingly, both proteins co-localized at the plasma membrane and this localization was enhanced upon HGF stimulation. Co-expression of GFP Gab1 with a mutant construct of STAM lacking the SH3 domain (STAMΔSH3) failed to target to the plasma

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membrane and no co-localization between both proteins was observed (Fig 7A). Staining of endogenous STAM was also performed to ensure that plasma membrane localization was not an artifact of over expression (Fig 7B).

As the localization of STAM to the plasma membrane has never been reported, I next addressed whether its binding partner, Hrs could also be targeted to the plasma membrane and found in a complex with Gab1. Co-expression of HA Gab1 and GFP Hrs revealed that Hrs does associate with Gab1, but unlike the Gab1-STAM interaction, requires Met activation (Fig 7C). Confocal microscopy imaging of GFP Gab1 with endogenous or transfected Hrs in MDCK cells (Fig 7D and E) showed Hrs staining at the plasma membrane, coincident with GFP Gab1 upon HGF stimulation.

The previous finding that Gab1 localizes to CDRs, prompted me to examine whether Hrs and STAM were also recruited to these structures upon HGF stimulation. Live cell imaging using both confocal microcopy and spinning disk confocal microscopy established that both GFP Hrs and mCherry STAM localize to areas of membrane ruffling with Gab1 and are also present on circular ruffles which form upon HGF stimulation (Fig 8Aand 9A). As a follow up to this observation, I am currently addressing several hypotheses for the role(s) of these proteins at the plasma membrane. Firstly, are Hrs and/or STAM required for circular ruffle formation itself and secondly, do they function to recruit RTKs to be internalized through circular ruffles.

To test the first hypothesis that Hrs and STAM are required for the formation of circular ruffles, I will score for circular ruffle formation (visualized by staining for actin) upon depletion of either protein singly or together with siRNA in MDCK cells. Conditions for efficient knock down of both Hrs and STAM are currently being established. Live cell imaging of a MDCK GFP Gab1 stable cell line transfected with mCherry STAMΔSH3, showed very weak membrane ruffling and limited GFP Gab1 localization to these ruffles in cells expressing both proteins, unlike surrounding cells expressing only GFP Gab1 (Fig 8B). This preliminary data would suggest that STAM may play a role in either Gab1 recruitment to, or retention in membrane ruffles. Membrane ruffling has been demonstrated to require new membrane from internal vesicles (19). Rab5, a small GTP binding protein which localizes to early endosomes, has previously been reported to be required for CDR formation (20, 21). Since Hrs and STAM are present on Rab5 positive endosomes, they may provide the regulation for endosome membrane fusion events through their association with Gab1 (22). Several reports have now demonstrated a role for Hrs in recycling, indicating that Hrs is capable of trafficking on endosomes to fuse with the plasma membrane (23, 24). If Hrs and STAM depletion reduce CDR formation, I will examine whether Hrs/STAM positive endosomes fuse with and supply the cell surface with membrane required for ruffling events using spinning disk confocal and TIRF (Total Internal Reflection Fluorescence) microscopy. (Both microscopes are available for use in the McGill Life Sciences Imaging Facility). Similarly, I will assay whether this fusion event is regulated through the association of Gab1 with Hrs and/or STAM. Alternatively, since STAM can associate with Gab1 at the plasma membrane, endosome-plasma membrane fusion events may be mediated through an interaction between an endosome associated Hrs molecule and a plasma membrane associated STAM. These hypotheses will be examined through a structure function approach using mutants of Hrs that uncouple it from the endosome (HrsΔFYVE) and from STAM (HrsΔCC2) and a mutants of STAM which uncouple it from Hrs (STAMΔCC) and from Gab1 (STAMΔSH3) (22, 25). I am currently mapping the domain(s) required in Hrs to associate with Gab1.

To date, no data exists on the constitution of phosphatidylinositols within the membrane of circular ruffles. If Hrs and STAM were to provide endosomal membrane for these structures, one prediction could be that the phosphatidylinositol, PI3P, found on endosomes would also be concentrated

in ruffles. To test this possibility, I will use a GFP Hrs-FYVE domain which specifically binds PI3P, as marker for this lipid.

Finally, an interaction between Hrs and Pak1 has previously been published by Sasaki *et. al.* (26) (Fig 9B). Since Pak1 is required for dorsal ruffle formation, I will examine if uncoupling Hrs from Pak1 affects Hrs recruitment to the plasma membrane and CDR formation.

To address whether STAM and/or Hrs function to recruit RTKs into circular ruffles for internalization, I will knock down STAM and Hrs proteins and assay for Met recruitment into CDRs by live cell microscopy using stable MDCK cell lines expressing mCherry Met with either GFP STAM or GFP Hrs. Co-localization of Met with STAM does occur at the plasma membrane in HeLa cells (Fig 8C) but localization with STAM on circular ruffles remains to be tested. Notably, a Gab1 mutant uncoupled from STAM, Gab1ΔPro5, is unable to enhance Met degradation, suggesting that STAM recruitment to Gab1 is required (Fig 2B). This Gab1 mutant is currently being assayed for its ability to localize to circular ruffles.

Both STAM and Hrs are differentially phosphorylated downstream from a wide range of RTKs and G-Protein coupled receptors and therefore may represent a common mechanism to induce receptor internalization through circular dorsal ruffles.

These data will be written up as a manuscript and submitted for publication by July 2007.

(4) Determine the consequence of altered Met subcellular localization in human breast cancer cell lines.

Breast cancer is a very heterogeneous disease made up of subtypes that have diverse clinical outcomes. In recent years, microarray data has greatly contributed in our knowledge of different breast cancer subtypes at a molecular level (27-30). These studies have analyzed a panel of primary breast tumors, and hierarchical clustering has reproducibly identified 3 main subtypes of tumors: the luminal (estrogen (ER) and progesterone (PR) receptor positive), basal (triple negative: ER/PER/Her2 negative) and the Her2 (ER/PR negative, Her2 amplification and/or overexpression) groups. The basal-type of breast cancers have received particular attention as they correlate with poor outcome. Furthermore, due to their "triple negative" nature, they currently lack the benefit of targeted therapy. These breast cancers also tend to express immunohistochemical markers that are normally found in the "basal" layer of a bi-layered, normal mammary epithelium which is the layer that is closer to the basement membrane. Several studies have provided indirect evidence that Met-overexpressing breast cancers are part of the basal subtype. Charafe-Jauffret E *et al*. performed microarray analysis on 31 breast cancer cell lines, and found that these segregated into basal, and luminal-like clusters, and that the Met receptor was a marker of the basal breast cancer cell lines (31). More recently, it was reported by the same group that Met is overexpressed in tumors with poor prognosis that also overexpress basal markers (ie: cytokeratin 5/6) (32).

Recent data from our lab suggests that in several breast cancer cell lines, Met is localized to intracellular compartments rather than at the plasma membrane. In such cases, this can result is ligand-independent activation and aberrant signaling of the receptor, that may now escape ligand-mediated downregulation through the endocytic pathway. Using a panel of 'basal type' breast cancer cell lines (BT-20, BT549, Hs578T, MDA-231, MDA-436, MDA-435s) (*27*), I will determine Met subcellular localization using established compartmental markers for endosomes, golgi network and the ER (Rab7, Rab9, M6PR, LAMP-1, 58K golgi protein, calnexin and calreticulin) by confocal microscopy. The mode

of Met entry into the cell (clathrin, caveolae or circular ruffles) will also be examined using assays I have previously established. The mechanism of Met internalization and cellular localization will be compared to the normal breast epithelial cell line, MCF10A. In cell lines where Met is aberrantly localized, I will examine the half-life of the receptor and its ability to induce Hrs phosphorylation as a read out for efficient late endosome-lysosomal sorting. Identification of any alteration in Met endocytosis and subcellular localization in these cell lines could then be correlated with the invasive potential of each line (27).

Finally, I will address whether Met receptors that exhibit altered subcellular localization preferentially activate a specific signaling pathway(s). Those receptors that display aberrant subcellular localization, stability and signaling in human breast cancer cell lines will be further examined for point mutations, by generating cDNA and using nested oligonucleotide primers within Met to obtain the sequence. The importance of any point mutations to Met function and regulation will be studied by inserting these into a wt Met and examining as described above.

I will be starting this task in the upcoming months and will finish these studies by the start of 2008.

KEY RESEARCH ACCOMPLISHMENTS

- ❖ The Gab1 scaffolding protein localizes to dorsal ruffles upon HGF stimulation and over expression of Gab1 induces enhanced formation of these structures.

- ❖ Gab1 has a novel role in Met downregulation by enhancing Met degradation.

- ❖ Gab1 mediated Met degradation occurs through a novel route of Met internalization into the cell, through dorsal ruffles.

- ❖ Hrs and STAM localize to the plasma membrane and dorsal ruffles with Gab1, signifying a novel function for these endocytic adaptor proteins.

REPORTABLE OUTCOMES
Manuscripts

Abella JV, Peschard P, Naujokas MA, Lin T, Saucier C, Urbe S and Park M,. Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol. Cell. Biol., 2005, Nov:25(21) 9632-45.

Abstracts (since award start date)

ABELLA JV., Parachoniak C., Zuo D., Park M., (May 2007). *A novel role for the Gab1 scaffolding protein in mediating internalization of the Met receptor tyrosine kinase through circular ruffles*. Poster to be presented at the Cold Spring Harbor Meeting on Phosphorylation, Signaling and Disease.

ABELLA JV., Frigault M., Park M., (July 2006). *The role of the Gab1 scaffold protein in downregulation of the Met receptor tyrosine kinase*. Poster at Gordon Research Conference on Lysosomes and Endocytosis.

Presentations
Abella JV. *Exploring new mechanisms for receptor tyrosine kinase downregulation.* Molecular Oncology Group seminar series. November 2006, McGill University, Montreal, Canada.

Cell lines

MDCK cell lines/populations stably expressing :
     shRNA targeting Gab1
     GFP Hrs.
     mCherry Gab1.
     mCherry Gab1 and GFP Hrs.
     mCherry STAM .
     mCherry STAMΔSH3.
     GFP Gab1 and mCherry STAM
     GFP Gab1 and mCherry STAMΔSH3.
     GFP Met and mCherry Gab1.
     GFP Met and mCherry STAM.

T47D cell populations stably expressing:
     Met Y1003F/N1358H
     Met Y1349/56F

HeLa cell lines stably expressing shRNA targeting Gab1.

CONCLUSION

How signal output is regulated by the mode of receptor internalization and subcellular localization, is still an area that we know little about. My work has focused on trying to understand how the Met receptor internalizes and traffics within the cell under normal conditions and how deregulation of the receptor can be caused by aberrant subcellular localization. I have previously demonstrated that deregulation of the Met receptor, through loss of Cbl mediated ubiquitination, results in a receptor that exhibits altered trafficking, escapes degradation and induces sustained signaling of the MAPK pathway, leading to cell transformation (2). The data presented in this report demonstrates a novel mode of entry into the cell for the Met receptor, mediated by the Gab1 scaffolding protein, through circular dorsal ruffles or CDRs. Further characterization of this pathway revealed that two endocytic adaptor proteins, Hrs and STAM, only known to function at the level of the sorting endosome, associate with Gab1 and are present on CDRs. This may represent a novel complex involved in targeting Met and perhaps other RTKs for internalization through CDRs. This pathway leads to enhanced degradation of the Met

receptor compared to that downstream from clathrin mediated internalization. Therefore deregulation of this pathway could lead to enhanced stability and signaling of Met and other RTKs. Future work will determine whether breast cancer cell lines which express high levels of Met have selected against this faster mode of RTK degradation. I will also determine whether the aberrant subcellular localization of Met that has been observed in these cell lines is due to mutations within the Met receptor, or a defect in the endocytic machinery, which would impact on all receptors utilizing this pathway.

Our understanding of the regulatory events involved in mediating the trafficking and degradation of Met and other RTKs, and how these are altered in human breast cancer will impact on our understanding of the molecular mechanisms of breast cancer.

"SO WHAT"

Treatment strategies that are currently used on breast cancer patients with EGFR family positive tumors, involve the use of monoclonal antibodies (mAbs) such as Herceptin against HER2 and Cetuximab against EGFR. These mAbs target the receptor at the plasma membrane and function by inducing ligand-independent receptor internalization and degradation through the endocytic pathway, terminating sustained receptor signaling (33). Our lab has observed aberrant localization of the Met receptor in several breast cancer cell lines, where the majority of the receptor is present within the cell and not at the plasma membrane. Hence, cases where RTKs become misslocalized to a subcellular location, will be refractory to such treatments targeted to the extracellular domain at the cell surface. In addition, the drug gefitinib (Iressa), a specific small molecule inhibitor of EGFR tyrosine kinase, was found to be effective in suppressing the survival and proliferation in some but not all non-small cell lung cancer (NSCLC) cell lines, by enhancing the rate of EGFR trafficking and degradation (34). Work by Ono's group demonstrated that the cell line insensitive to gefitinib treatment harbored a defect in the endocytic machinery, preventing efficient degradation (34). The concentration of ligand available to receptors may also influence the endocytic route taken and therefore the signaling outcome (5). The hypothesis that deregulation of RTKs through altered endocytosis, is clearly being validated now with work from several groups reporting altered EGF and Met receptor trafficking in cancer cells (35, 36). Thus treatment strategies to induce degradation through the normal endocytic pathway will be ineffective in situations where the trafficking pathway itself is deregulated. It is therefore crucial to understand how receptors traffic and to identify the subcellular locations within the cells where aberrant RTK signaling is occurring. This will allow for the design of potentially more effective drug treatments that target the receptor at the appropriate cellular location.

Targeted therapies against the Met receptor are in development and studies suggest that such a drug would hold much promise in cancer treatment. The Met receptor is implicated in all stages of cancer progression, including proliferation, invasion and metastasis. It is also expressed in endothelial cells, and would allow therapies to not only target cancer epithelium, but the tumor microenvironment as well, which is believed to be important in tumorigenesis. Studies on Met receptor trafficking, subcellular localization and deregulated signaling will thus contribute to the design of effective Met-targeted drugs.

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Figure 1

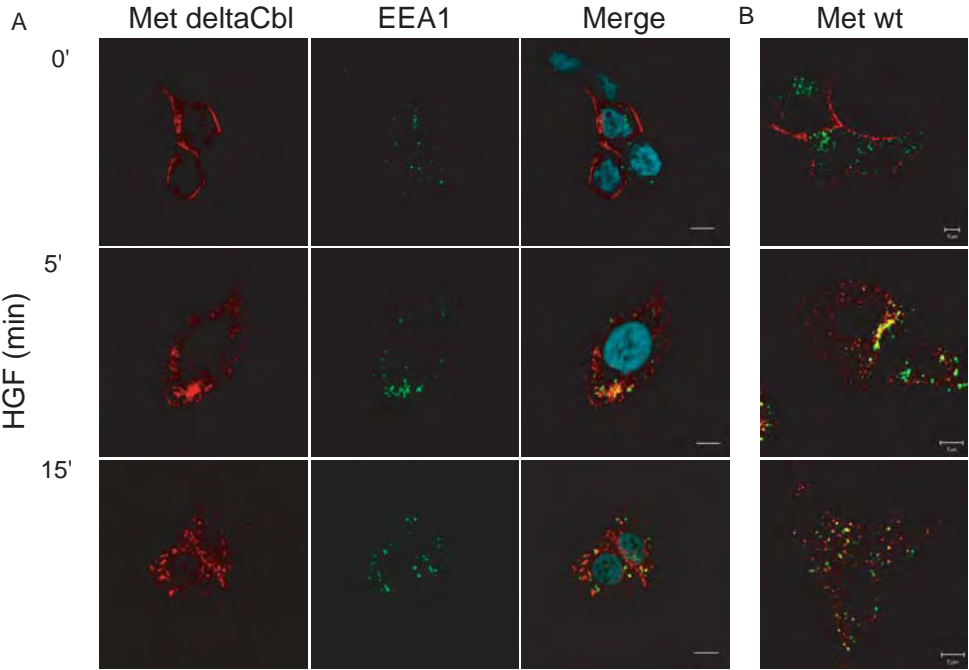|	Met deltaCbl	EEA1	Merge		Met wt
A				**B**	

HGF (min)

Figure 1. The Met Receptor Tyrosine Kinase does not require Grb2 or Cbl to internalize in to cells. T47D breast cancer epithelial cell lines stably expressing wt Met or a Met mutant uncoupled from both Grb2 and Cbl (Met Y1003F/N1358H), were stimulated with 0.6nM HGF for the indicated time. Cells were then fixed and stained for Met (red) and the early endosome marker EEA1(green) and Met internalization was analysed with a Ziess 510 Meta confocal microscope . Bar represents 5µm.

Figure 2

A

	Vector	HA-Gab1	HA-Gab1
HGF (hrs)	0 1 2 4	0 1 2 4	0 1 2 4

Met
HA

D

	HA-Gab1
HGF (hrs)	0 2 4 0 2 4

Met
HA
Actin

Boiling Lysis lysates

B

	Vector	HA-Gab1ΔPH	HA-Gab1ΔPro5
HGF (hrs)	0 1 2 4	0 1 2 4	0 1 2 4

Met
HA

G

0   0.5   1   2   ug HA Gab1

Met
HA
Actin

TCL

C

	MDCK wt	MDCK HA-Gab1
HGF (hrs)	0 1 2 4	0 1 2 4

Met
HA

F

Vector   HA Gab1   HA Gab1DPH   HA Gab1DPro5

HGF   0 5   0 5   0 5   0 5

IP Met
250 —
150 —
P4D1

150 —
Met

TCL
Met
HA

Figure 2. Gab1 overexpression enhnaces HGF dependent Met degradation. (A and B) HeLa cell lysates expressing vector of HA Gab1 and Gab1 mutants through transient trafection were immnuoblotted for Met and Ha Gab1 levels. GabΔPH, lacks the pleckstrin homology domain and GabΔPro5 lacks the atypical Proline rich motif 5. (C) MDCK cells with or without stable expression of HA Gab1, were stimulated and processed as in A. (D) HeLa cells were stimulated with HFG as in A, but lysed in 2% SDS boiling lysis buffer. (G) HeLa cells were transiently transfected with increasing amount of Ha Gab1 and lysated were probed for endogenous Met levels. (F) HeLa cells transiently transfected with HA Gab1 constructs were stimulated for 5 minutes with HGF and lysated were immunoprecipitated for endogenous Met and probed for ubiquitin.
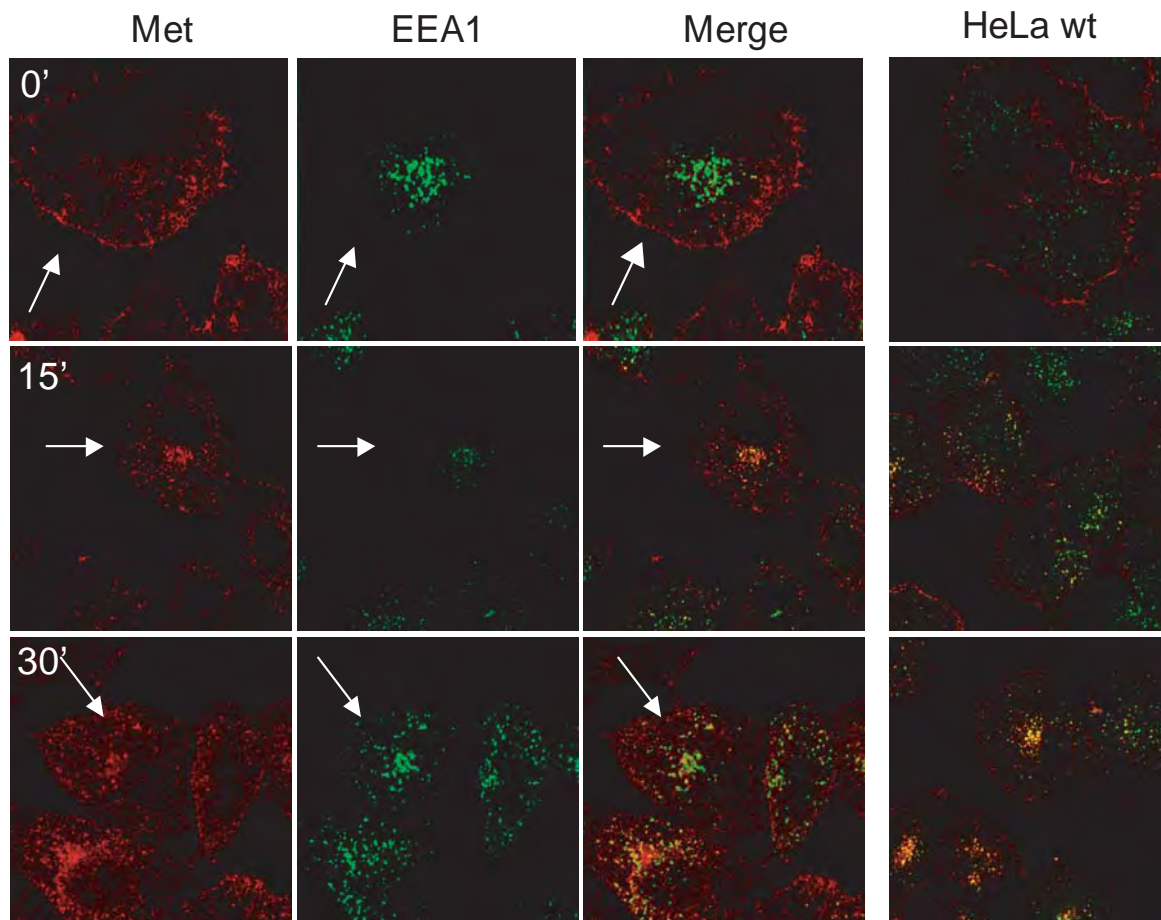
Figure 3

Figure 3. HA Gab1 enhances Met trafficking to a perinuclear compartment. HeLa Cells transfected with HA-Gab1 or vector (Hela wt), stimulated for indicated times with 0.6nM HGF, fixed with 2% PFA and visualized by indirect immunofluorescence (anti-EEA1 and anti-Met) and confocal microscopy. 100X mag, 1.5Xzoom. White arrows represent HA Gab1 transfected cells.
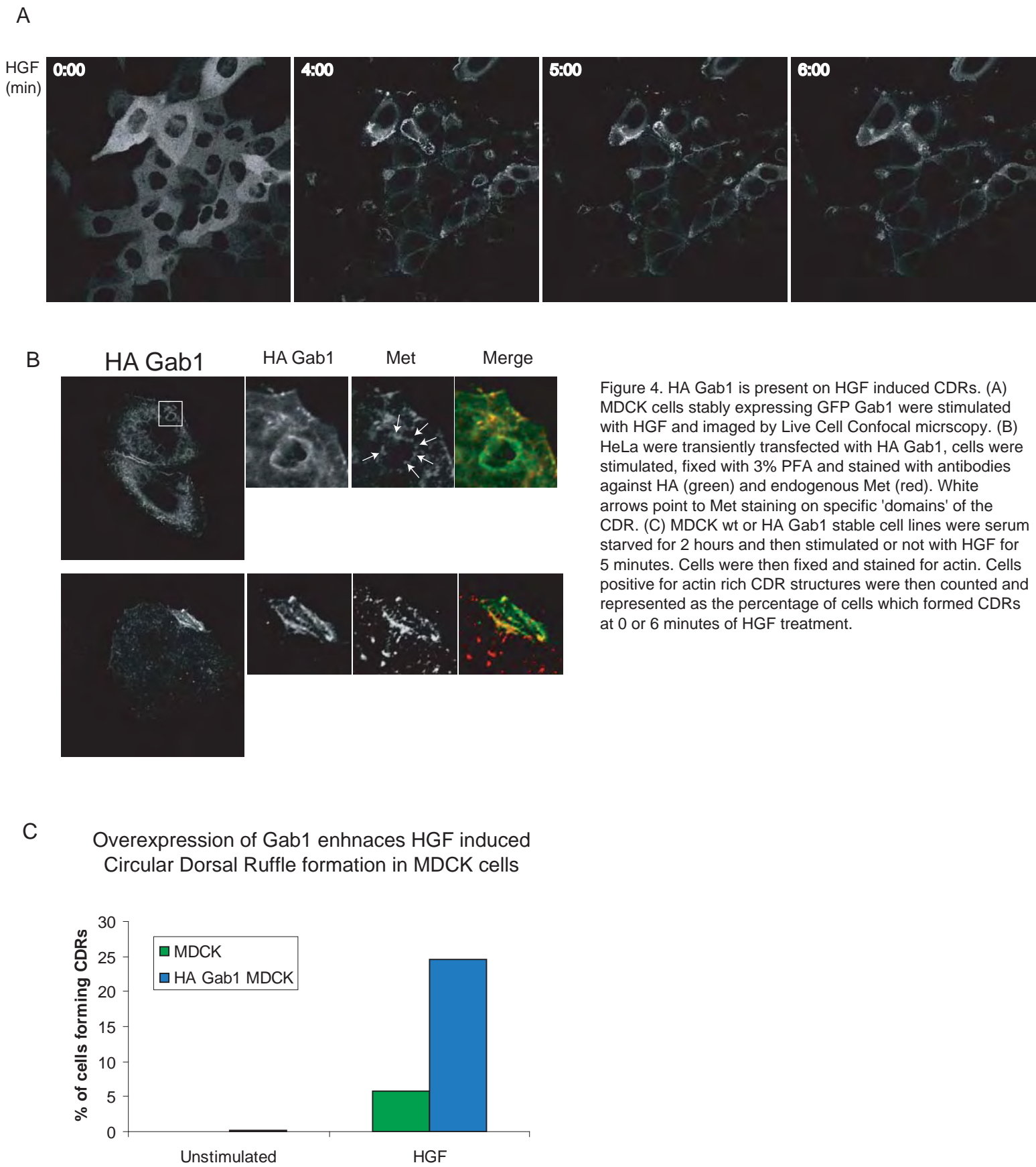
Figure 4

A

HGF (min)   0:00   4:00   5:00   6:00

B

HA Gab1   HA Gab1   Met   Merge

Figure 4. HA Gab1 is present on HGF induced CDRs. (A) MDCK cells stably expressing GFP Gab1 were stimulated with HGF and imaged by Live Cell Confocal micrscopy. (B) HeLa were transiently transfected with HA Gab1, cells were stimulated, fixed with 3% PFA and stained with antibodies against HA (green) and endogenous Met (red). White arrows point to Met staining on specific 'domains' of the CDR. (C) MDCK wt or HA Gab1 stable cell lines were serum starved for 2 hours and then stimulated or not with HGF for 5 minutes. Cells were then fixed and stained for actin. Cells positive for actin rich CDR structures were then counted and represented as the percentage of cells which formed CDRs at 0 or 6 minutes of HGF treatment.

C

Overexpression of Gab1 enhnaces HGF induced Circular Dorsal Ruffle formation in MDCK cells

Figure 5

A

myc Pak1

HA Gab1

HA Gab1 + Met

IP myc
HA Gab1
myc Pak1

TCL
HA Gab1
myc Pak1

B

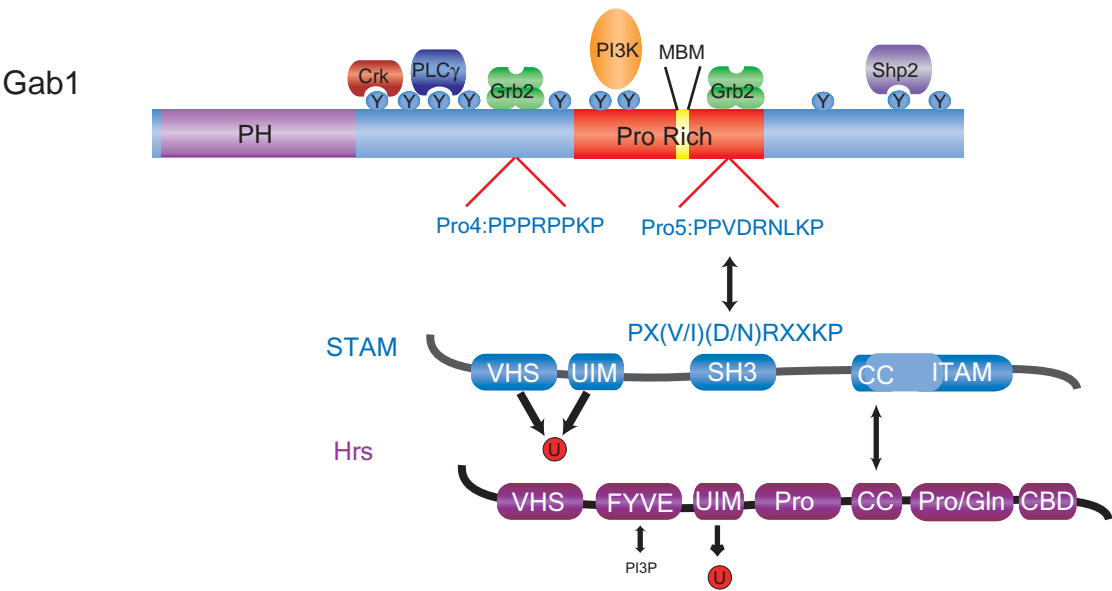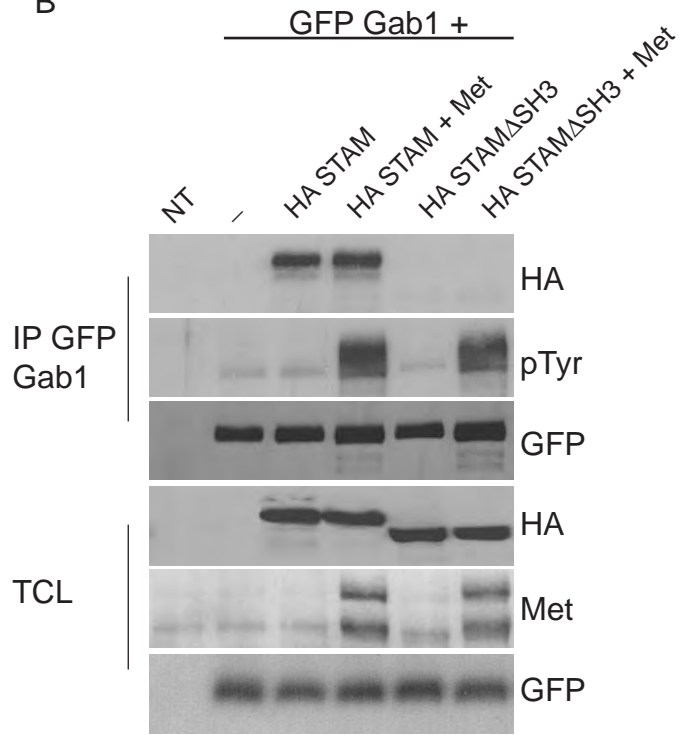| HGF (hrs)	Vector				myc Pak1				
	0	1	2	4	0	1	2	4	

Met

myc

Actin

Figure 5. Pak1 associates with Gab1 and also enhances Met receptor degradation. (A) 293 cells were transiently transfected with HA Gab1, myc Pak1 and Met (combinations indicated) and lysates were immunoprecipitated for myc-Pak1 and immunoblotted for HA Gab1. (B) HeLa cells were transiently trasnfected with vector or myc-Pak1 and stimulated with 0.6 nM HGF for the indicated times. Lysates were then probed for endogenous Met levels and myc Pak1 levels.

Figure 6
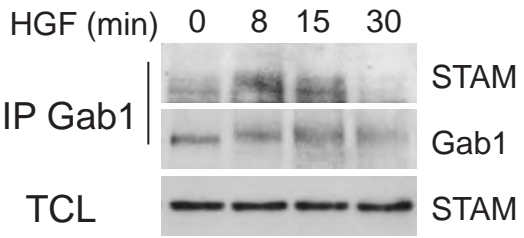
A

B

C

D

Figure 6. Gab1 and STAM interact through an SH3-Proline dependent manner. (A) Cartoon model of Gab1, STAM and Hrs domain structures. (B and C) 293 cells transiently transfected with full length and mutant GFP Gab1 and HA STAM. B, GFP Gab1 was immunoprecipiated from lysated and immunoblotted for HA STAM. C, GFP Gab1 mutants were immunoprecipitated and immunobloted for HA STAM. (D) HeLa cells were stimulated with 0.6nM HGF for the indicated time and endogenous Gab1 was immunoprecipiated from lysated and immunoblotted for endogenous STAM.

Figure 7

A

| GFP Gab1 | HA STAM | Merge | GFP Gab1 | HA STAMΔSH3 | Merge |

0' / 15'

B

HA Gab1 / STAM / Merge

C

GFP Hrs

HA-Gab1 / HA-Gab1 + Met

IP GFP Hrs — HA, GFP

TCL — HA, GFP, Met

D

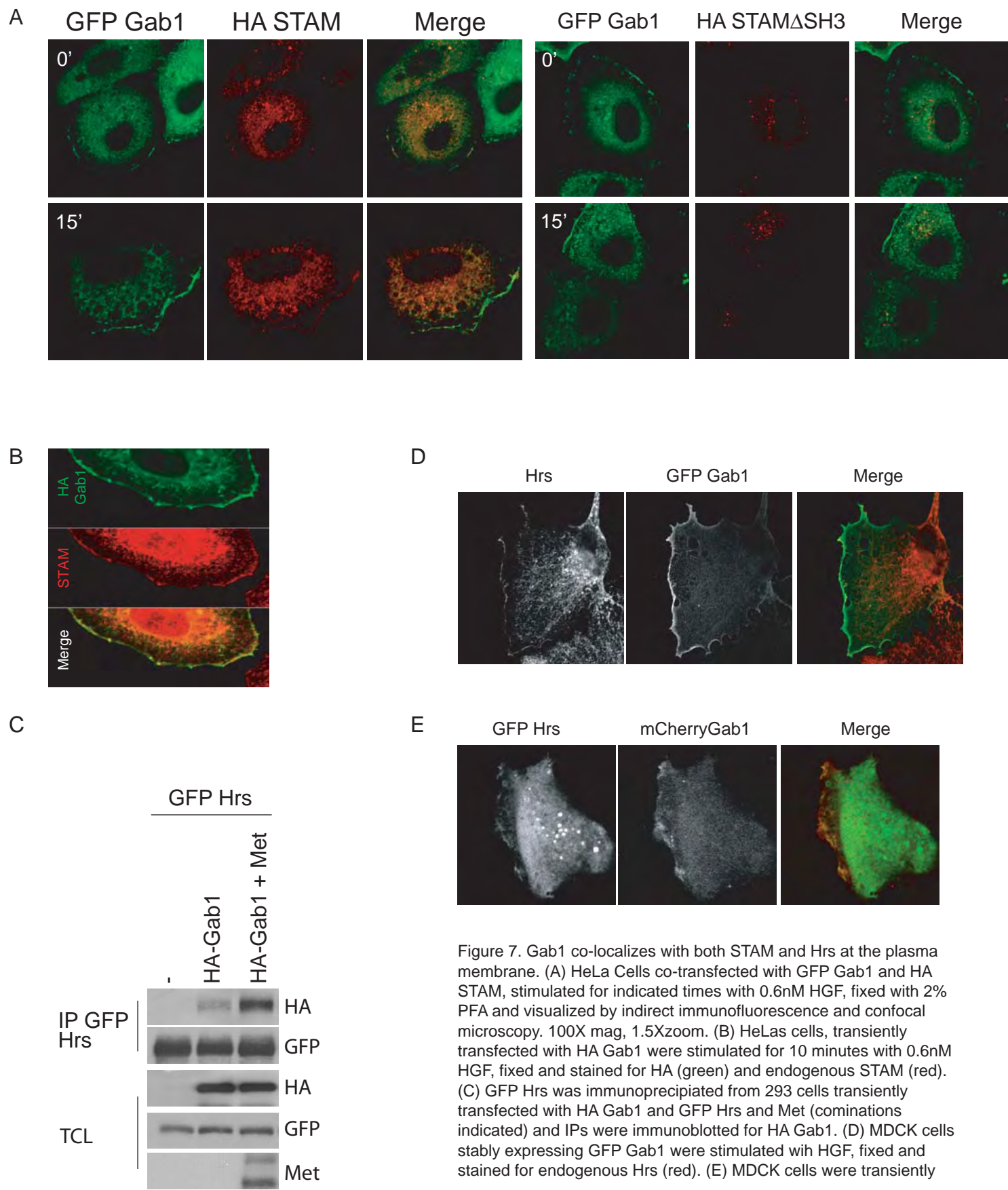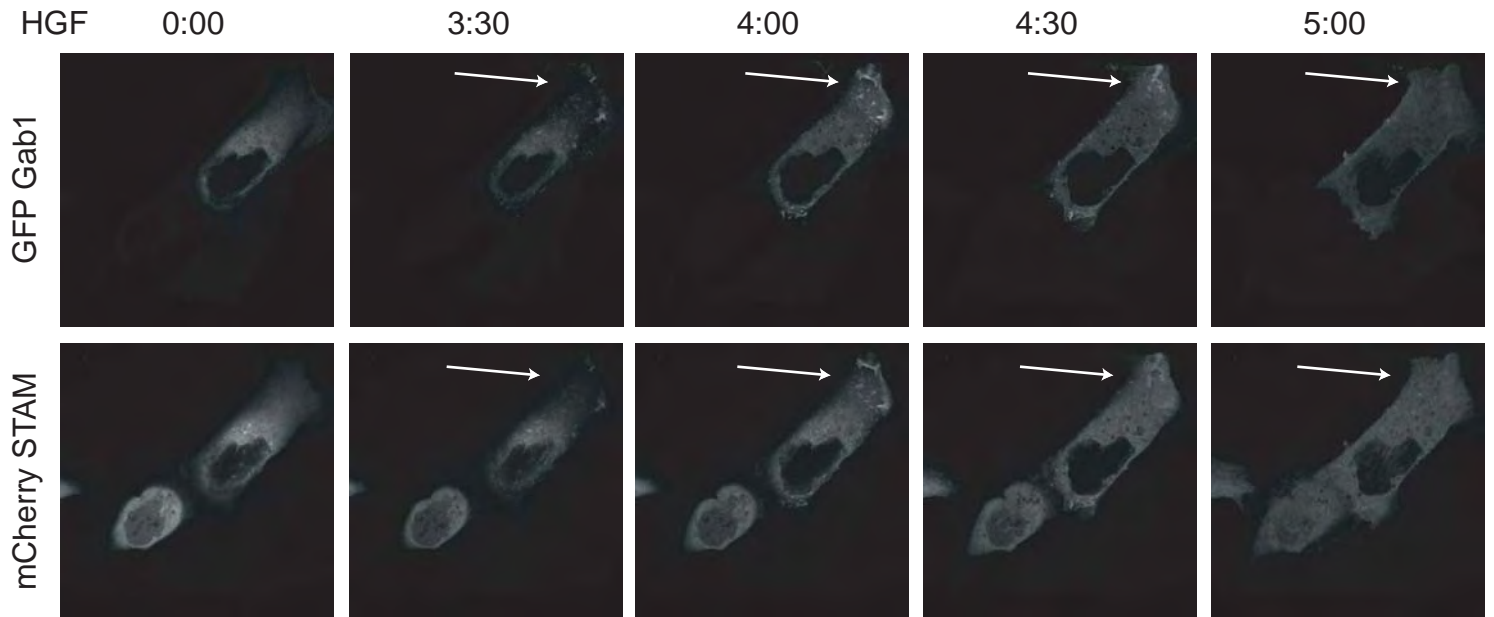| Hrs | GFP Gab1 | Merge |

E

| GFP Hrs | mCherryGab1 | Merge |

Figure 7. Gab1 co-localizes with both STAM and Hrs at the plasma membrane. (A) HeLa Cells co-transfected with GFP Gab1 and HA STAM, stimulated for indicated times with 0.6nM HGF, fixed with 2% PFA and visualized by indirect immunofluorescence and confocal microscopy. 100X mag, 1.5Xzoom. (B) HeLas cells, transiently transfected with HA Gab1 were stimulated for 10 minutes with 0.6nM HGF, fixed and stained for HA (green) and endogenous STAM (red). (C) GFP Hrs was immunoprecipiated from 293 cells transiently transfected with HA Gab1 and GFP Hrs and Met (cominations indicated) and IPs were immunoblotted for HA Gab1. (D) MDCK cells stably expressing GFP Gab1 were stimulated wih HGF, fixed and stained for endogenous Hrs (red). (E) MDCK cells were transiently
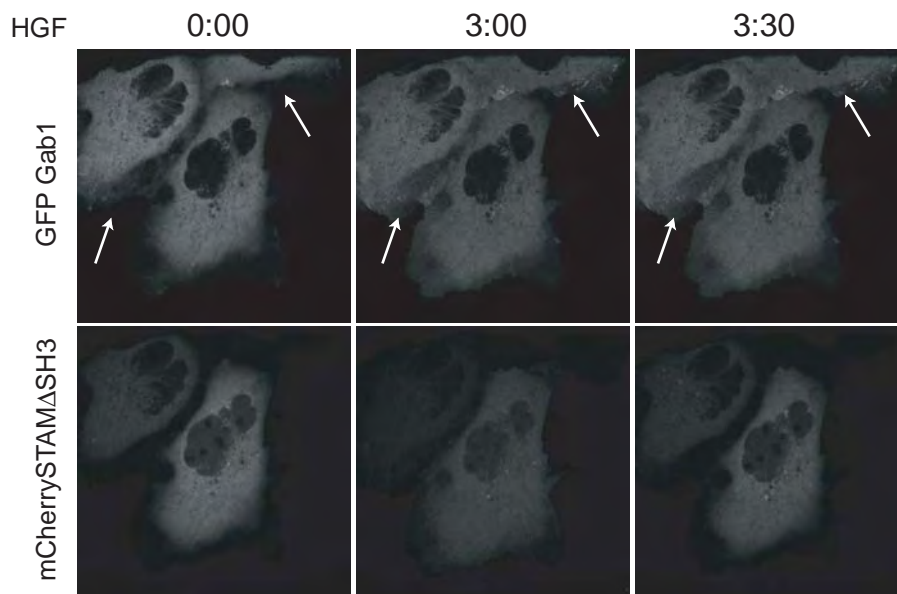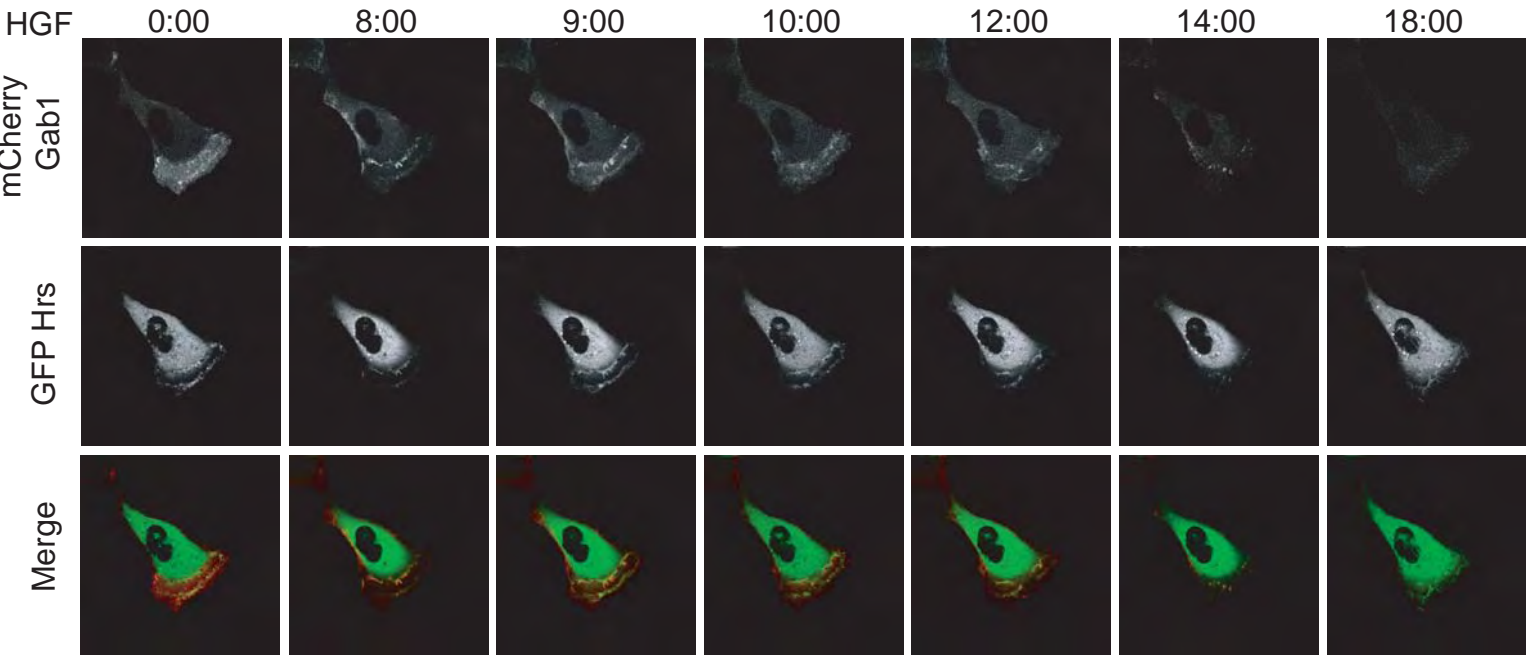
Figure 8

A

B

Figure 8. STAM and Gab1 localize to Circular Dorsal Ruffles in an HGF dependent manner and requires the SH3 domain of STAM. (A) Live cell spinning disk confocal microscopy images time lapse images of MDCKs transfected with mCherry STAM or mCherry STAMΔSH3 (B) and GFP Gab1 stimulated with HGF. (A) arrows indicate place of CDR formation. (B) Arrows indicate GFP Gab1 positive ruffles in surrounding cells unstranfected with mCherry STAMΔSH3.
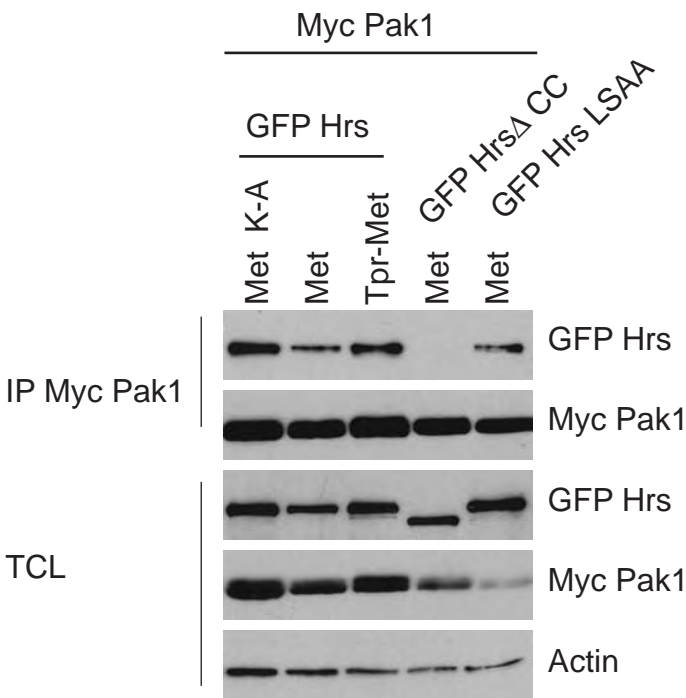
Figure 9

A

B

Figure 9. (A) Time lapse images of GFP Hrs and mCherry Gab1 in MDCKs upon HGF stimulation imaged through confocal microscopy show both proteins present on a large CDR sructure. (B) 293 cells transiently transfected with myc Pak1 and GFP Hrs and Hrs mutants, lysates were immunoprecipitated for myc Pak1 and immunoblotted for GFP Hrs.